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A new multiplex method for the diagnosis of peroxisomal disorders allowing simultaneous determination of plasma very-long-chain fatty acids, phytanic, pristanic, docosahexaenoic and bile acids by high-performance liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry

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ABSTRACT

Peroxisomal disorders (PDs) present with wide phenotypic variability. An appropriate diagnosis requires a complete analysis of peroxisomal metabolites.

We developed a multiplex LC-MS/MS method, using atmospheric pressure chemical ionization allowing the simultaneous determination in plasma of very-long-chain fatty acids, phytanic, pristanic, docosahexaenoic acids and di- and tri-hydroxycolestanoic bile acids.

Two hundred microliters of plasma extracted with acetonitrile and 200 μl extracted with hexane after an acid hydrolysis were combined, evaporated, dissolved in 10 μl of methanol and analyzed.

The acquisition was in negative-ion mode using multiple reaction monitoring.

The method was validated analytically and clinically. Linearity was 0.1–200 μmol/l for docosanoic, cis-13-docosenoic, tetracosanoic, cis-15-tetracosenoic and phytanic acids; 0.01–10 μmol/l for hexacosanoic acid; 0.02–20 μmol/l for di-hydroxycolestanoic, tri-hydroxycolestanoic and pristanic acids; 0.3–300 μmol/l for docosahexaenoic acid. Intra-day and inter-day CVs were below 3.88 and 3.98 respectively for all compounds. Samples from patients with known peroxisomal disorders were compared with controls and the method allowed to confirm the diagnosis in all subjects with a 100% sensitivity.

The advantage of this multiplex method is to allow in a single chromatographic run the simultaneous determination of a large number of peroxisome biomarkers with a simple preparative phase without derivatization.

Abbreviations: PDs, peroxisomal disorders; LC-MS/MS, liquid chromatography-tandem mass spectrometry; CV, coefficient of variation; VLCFAs, very-long-chain fatty acids; Pri, pristanic acid; PUFAs, polyunsaturated fatty acids; DHA, docosahexaenoic acid; Phy, phytanic acid; PBDs, peroxisomal biogenesis disorders; GC–MS, gas chromatography–mass spectrometry; ESI-MS/MS, electrospray ionization tandem mass spectrometry; UPLC, ultra-performance liquid chromatography; DAABD-AE, 4-[2-(*N*,*N*-dimethylamino) ethylaminosulfonyl]-7-(2-aminoethylamino)- 2,1,3-benzoxadiazole; HPLC-MS/MS, high performance liquid chromatography-tandem mass spectrometry; APCI, atmospheric pressure chemical ionization; [^{2}H3]-Phy, [$^2H_3$]- phytanic acid; [$^2H_3$]-Pri, [$^2H_3$]-pristanic acid; DHCA, 3α,7α-dihydroxy-5β-cholestan-26-oic acid; THCA, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; [$^2H_3$]-DHCA, 27,27,27[$^2H_3$]-3α,7α-dihydroxy-5β-cholestan-26-oic acid; [$^2H_3$]-THCA, 27,27,27[$^2H_3$]-3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; [$^2H_4$]-C22:0, 3,3,5,5[$^2H_4$]-docosanoic acid; [$^2H_4$]-C24:0, 3,3,5,5[$^2H_4$]-tetracosanoic acid; [$^2H_4$]-C26:0, 3,3,5,5[$^2H_4$]-hexacosanoic acid; C22:1, cis-13-docosenoic acid; C24:1, cis-15-tetracosenoic acid; C22:0, docosanoic acid; C24:0, tetracosanoic acid; C26:0, hexacosanoic acid; ACN, acetonitrile; ERNDIM, European Research Network for Evaluation and Improvement of Screening Diagnosis and Treatment of Inherited Disorders of Metabolic; DP, Declustering Potential; CXP, Collision Cell Exit Potential; CE, Collision Energy; MRM, multiple reaction monitoring; X-ALD, X-linked adrenoleukodistrophy; ARF, adult Refsum disease; RT, retention time; SD, standard deviation; R^2, coefficient of linear regression; LLOD, lower limit of detection; S/N, signal-to-noise ratio; LLOQ, lower limit of quantification.

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1. Introduction

Peroxisomes are subcellular organelles DNA-free, bounded by a single membrane and ubiquitously presents in eukaryotes. They are extremely important for human health and development, because catalyze many essential metabolic functions in hydrogen peroxide and lipid metabolism [1]. The main functions of peroxisomes are: 1) β-oxidation of very-long-chain fatty acids (VLCFAs) and pristanic acid (Pri, C19:0 branched); 2) synthesis of polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA, C22:6ω-3); 3) conversion of cholesterol into bile acids; 4) α-oxidation of phytanic acid (Phy, C20:0 branched); 5) synthesis of etherphospholipids, including plasmalogens. Moreover, peroxisomes participate in purine, polyamine, glyoxylate and amino acids metabolism [2].

Peroxisomal disorders (PDs) are currently classified into two main groups: the peroxisomal biogenesis disorders (PBDs) and the disorders of single peroxisomal function. PBDs include Zellweger spectrum disorders (Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease) and rhizomelic chondrodysplasia Type 1 and 5. The disorders of single peroxisomal function result in defects of a single peroxisomal enzyme or transporter and can be sub-classified into disorders of fatty acid beta-oxidation (X-linked adrenoleukodystrophy, acyl-CoA oxidase deficiency, D-bifunctional protein deficiency, sterol-carrier-protein X deficiency, 2-methylacyl-CoA racemase deficiency, peroxisomal membrane protein 70 deficiency), disorders of etherphospholipid biosynthesis (rhizomelic chondrodysplasia puncatata Type 2, 3, 4), disorders of fatty acid alpha-oxidation (Refsum disease), disorders of glyoxylate metabolism (hyperoxaluria Type 1, isolated glycolic aciduria), disorders of bile acid synthesis (bile acid-CoA: amino acid N-acyltransferase deficiency) and disorders of H_2O_2-metabolism (acatalasemia) [2–5].

Clinically, PDs cause severe neurological symptoms combined with ocular and hearing signs, dimorphisms, liver disease and skeletal abnormalities [4].

Due to the wide heterogeneity of PDs, for a correct diagnosis the signs and symptoms of each patient should be evaluated together with the analysis in blood of a wide panel of biomarkers, which includes VLCFAs, Phy, Pri, DHA, DHCA, THCA bile acids and plasmalogens [6].

Determination of VLCFAs, Phy and Pri, is traditionally carried out by gas chromatography–mass spectrometry (GC–MS), however a few LC-MS/MS methods were developed in the years [7–9]. In 2003, Johnson improved his original electrospray ionization tandem mass spectrometry (ESI-MS/MS) method [10] by including a liquid chromatography step and a second derivatization. This new method allowed the simultaneous determination of Pri, Phy, and VLCFAs, however required a multistep derivatization and a long chromatographic time [11]. In 2008, Al-Dirbashi developed an ultra-performance liquid chromatography (UPLC)-MS/MS method for the simultaneous analysis of VLCFAs, Phy, and Pri that involves derivatization with 4-[2-(*N*, *N*-dimethylamino) ethylaminosulfonyl]-7-(2-aminoethylamino)-2, 1, 3-benzoxadiazole (DAABD-AE) [12].

Here we report an easy high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method for the simultaneous determination in plasma of Phy, Pri, DHA, bile acids and VLCFAs (saturated and monounsaturated) requiring no derivatization and a simple sample preparation. The method used stable isotope-labelled internal standards and atmospheric pressure chemical ionization (APCI) as alternative ionization technique. This multiplex method allows a rapid diagnostic screening of peroxisomal diseases in a single chromatographic run with the simultaneous determination of most peroxisomal biomarkers.

2. Materials and methods

2.1. Reagents

[$^2H_3$]-phytanic acid ([$^2H_3$]-Phy), [$^2H_3$]-pristanic acid ([$^2H_3$]-Pri), Phy, Pri, 3α,7α-dihydroxy-5β-cholestan-26-oic acid (DHCA), 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA), 27,27,27[$^2H_3$]-3α,7α-dihydroxy-5β-cholestan-26-oic acid ([$^2H_3$]-DHCA), 27,27,27-[$^2H_3$]-3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid ([$^2H_3$]-THCA), 3,3,5,5[$^2H_4$]-docosanoic acid ([$^2H_4$]-C22:0), 3,3,5,5[$^2H_4$]- tetracosanoic acid ([$^2H_4$]-C24:0), 3,3,5,5[$^2H_4$]-hexacosanoic acid ([$^2H_4$]-C26:0) were purchased from VU Medical Center Metabolic Laboratory (Amsterdam, The Netherlands), cis-13-docosenoic acid (C22:1), cis-15-tetracosenoic acid (C24:1), cis-4,7,10,13,16,19-docosahexaenoic acid (DHA), docosanoic acid (C22:0), tetracosanoic acid (C24:0), hexacosanoic acid (C26:0), methanol, n-propanol and ammonium acetate, hydrochloric acid 37% were purchased from Sigma-Aldrich (Steinheim, Germany). HPLC grade acetonitrile (ACN) and water were purchased from Romil Ltd. (The Source Convent Drive Waterbeach Cambridge, United Kingdom). Quality control samples were from ERNDIM (European Research Network for Evaluation and Improvement of Screening, diagnosis, and Treatment of Inherited Disorders of Metabolic, http://www.erndimqa.nl/).

2.2. Preparation of standard solutions

"Stock solutions" 1 mmol/l of Phy, Pri, DHCA, THCA, C22:1, C24:1, C22:0, C24:0, C26:0, DHA and internal standard [$^2H_3$]-Phy, [$^2H_3$]-Pri, [$^2H_3$]-DHCA, [$^2H_3$]-THCA, [$^2H_4$]-C22:0,[$^2H_4$]-C24:0, [$^2H_4$]-C26:0 were prepared in chloroform-methanol 50/50 (v/v) and stored at −80 °C. The "internal standard mix solution 1" (50 μmol/l [$^2H_4$]-C22:0, 50 μmol/l [$^2H_4$]-C24:0, 5 μmol/l [$^2H_4$]-C26:0, 5 μmol/l [$^2H_3$]-Phy, 5 μmol/l [$^2H_3$]-Pri) and the "internal standard mix solution 2" (5 μmol/l [$^2H_3$]-DHCA, 5 μmol/l [$^2H_3$]-THCA) were prepared in methanol by scalar dilution of the 1 mmol/l stock solution.

2.3. Sample treatment procedure

VLCFAs, Pri, Phy and DHA required a distinct sample extraction procedure than DHCA and THCA bile acids because the former need to be released from phospholipid cholesterol esters while the latter are freely soluble in plasma. DHCA and THCA are subject to degradation in the hot acid environment.

VLCFAs, Pri, Phy and DHA extraction procedure: 200 μl of plasma were added to 200 μl of "internal standard mix solution 1" dried under a nitrogen stream and left under stirring for 15 min in a clear, screw-capped 16x100mm glass tube. Samples were hydrolyzed by incubating at 100 °C for 1 h with 3 ml of ACN/37% hydrochloric acid (5:1, v/v). After cooling down to room temperature, samples were extracted with 3 ml of hexane, vortex-mixed for 20 s and centrifuged at 3500 rpm for 5 min.

Bile acids extraction procedure: 200 μl of plasma were added to 200 μl of "internal standard mix solution 2" dried under a nitrogen stream and left under stirring for 15 min in a microfuge tube. 1 ml of ACN was added and samples were vortexed vigorously for 30 s and centrifuged at 12,000 rpm for 15 min.

At the end, both extract were merged together, transferred in a glass tube and evaporated.

Dry residue was dissolved in 200 μl of methanol and 10 μl were injected into the mass spectrometer.

2.4. Liquid chromatography-mass spectrometry

Chromatographic separation was obtained with a 150 × 3.00 mm 110A 3 μ Gemini-NX C18 column (Phenomenex, Castel Maggiore, Italy) and performed on an Agilent series 1200 pump and autosampler (Agilent technologies Inc., Wilmington, DE, USA). Mobile phase A was H_2O containing 20 mmol/l ammonium acetate and mobile phase B was methanol/n-propanol 90/10 (v/v). 10 μl of sample were injected onto the column. Chromatographic separation of metabolites was achieved with a flow rate of 500 μl/min in isocratic conditions (10% of